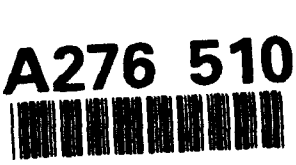

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TECHNICAL REPORT 9206

EVALUATION OF CHLORINE DIOXIDE GENERATED IN-SITU FOR DISINFECTION OF WATERBORNE MICRO-ORGANISMS

STEPHAN A. SCHAUB
HELEN T. HARGETT
KURT I. KAMRUD
CHARLES R. STERLING
MARILYN M. MARSHALL

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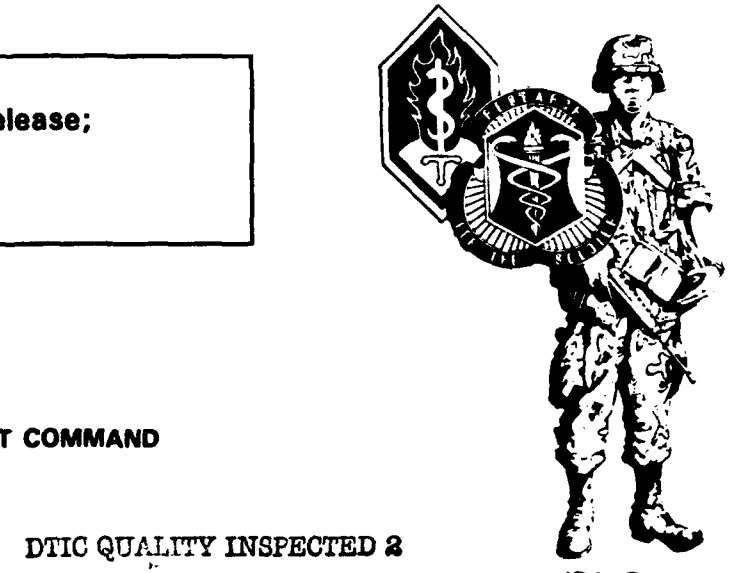
U.S. ARMY BIOMEDICAL RESEARCH & DEVELOPMENT LABORATORY
Fort Detrick
Frederick, MD 21702-5010



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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Tests were conducted with chlorine dioxide (ClO_2) field "burst packets" to determine their capability to produce chlorine dioxide from precursors and to determine if the disinfectant is appropriate for military field applications. Chlorine dioxide produced from citric acid and sodium chlorite was evaluated on Klebsiella terrigena, echovirus 7 and Cryptosporidium parvum oocysts. Waters used to challenge disinfection were distilled deionized, tap and worst case (containing humic acids, inorganic salts, high turbidity and high pH). Animal (neonatal mouse) infectivity tests were conducted at the University of Arizona with C. parvum oocysts after exposure to chlorine dioxide. Results indicate that the chlorine dioxide packets are effective for disinfection of bacterial and virus challenges with less than 10 minutes contact, but are ineffective for C. parvum oocysts with 30 minutes contact.

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PREFACE

The studies reported herein were performed in collaboration with Dr. Charles R. Sterling and Marilyn M. Marshall, Department of Veterinary Science, University of Arizona, Tucson, AZ, under work order DAMD17-90-M-S981.

INTRODUCTION

The U.S. Army is interested in providing soldiers with an improved field drinking water disinfectant. It is essential that a new disinfectant be capable of treating bacteria, viruses, and protozoan cysts and not produce waters that have adverse organoleptic properties, such as foul tastes and odors. Also, an improved disinfectant should be easy to use and provide rapid treatment, thus reducing the "wait time" needed before the water is safe to drink.

By direction of the Department of Defense Water Resources Management Action Group (WRMAG), an activity sponsored by the Army Deputy Chief of Staff for Logistics, the U.S. Army Biomedical Research and Development Laboratory (USABRDL) has been tasked to conduct studies on the disinfection efficacy of chlorine dioxide under conditions of field challenge for typical waterborne enteric microorganisms (bacteria, viruses, and protozoan cysts) to evaluate its use as a disinfectant for field water by the individual soldier.

Typically, chlorine dioxide (ClO_2) is used as a substitute for or an adjunct to the use of free available chlorine in treatment of municipal drinking water. Unlike chlorine, chlorine dioxide does not react with organic materials in water to produce carcinogenic trihalomethanes. It is also capable of reducing offensive tastes and odors. For the present study, prototype packets for generating chlorine dioxide in the field were provided by BIO-CIDE International, Inc. of Norman, OK. The packets are designed with two compartments, one containing a solution of sodium chlorite and other oxychlorine precursors and the second containing citric acid solution for pH adjustment. Chlorine dioxide is produced when the contents are combined by forcing the citric acid solution into the adjacent compartment. Because the rate of formation of chlorine dioxide under these conditions and the reproducibility of chlorine dioxide formation among the packets had not been established, preliminary characterization experiments were performed at USABRDL to establish a reliable and reproducible method for the test procedures. Inhouse disinfection efficacy studies were performed in general accordance with U.S. Environmental Protection Agency (USEPA) guidance.[1]

Collaborative research efforts between USABRDL and the University of Arizona, Department of Veterinary Science, were also conducted to determine the effectiveness of chlorine dioxide for disinfection of Cryptosporidium parvum oocysts, utilizing residual animal infectivity as the measure of effectiveness. These studies were initiated when it became apparent from USABRDL inhouse efforts that the oocysts remained intact and showed no morphological deterioration microscopically after chlorine dioxide exposure. Operational and testing procedures were identical to those used during USABRDL tests except that the strain of Cryptosporidium oocysts used was that provided by the University of Arizona.

MATERIALS AND METHODS: INHOUSE STUDY

1. PHYSICAL/CHEMICAL WATER CHARACTERISTICS

The tests to determine the effectiveness of chlorine dioxide examined a range of physical/chemical challenge conditions in various disinfectant free waters (Table 1).

TABLE 1. WATER QUALITY CHALLENGE CONDITIONS

Type	Test water conditions Temp.(C)	pH	Turbidity NTU	TDS mg/L	TOC mg/L
DF[a]	5 and 24	NA[b] and 9[c]	≤5.0	50-500	≤5.0
Tap	5 and 24	NA and 9	≤5.0	50-500	≤5.0
WC2[d]	5 and 24	NA and 9	≥30.0	≥1500	≥10.0

a = demand free
b = not adjusted to maintain original pH
c = adjusted to maintain pH 9.0
d = worst case

a. Glassware and Reagents. Halogen demand-free water was prepared from distilled deionized water by addition of Chlorox™ to provide free available chlorine (10 mg/L). After storage in the dark at room temperature for 24 hours, the water was dechlorinated by exposure to sunlight. This water was then used to rinse glassware and to prepare buffer solutions. All glassware items for these tests were washed, cleaned in a solution of sulfuric acid containing No-Chromix™, washed again in detergent, rinsed with distilled water, soaked overnight in a tank filled with distilled deionized water containing 20 mg/L of chlorine, and rinsed in halogen demand-free water. The glassware was sterilized in a dry heat oven.

b. Test Waters. Water characteristics, pH and temperatures were adjusted for each run according to the set of conditions being tested. For unadjusted pH tests, the beakers were filled with 1 liter of challenge water. To maintain pH 9.0, the beakers were filled with 900 mL of challenge water and 100 mL of halogen demand-free 0.05 M borate buffer. In each experiment four beakers of challenge water were prepared; two beakers with all challenge organisms for replicate chlorine dioxide tests, one control beaker for determining viability of all challenge organisms, and one control beaker of water for measuring chlorine dioxide disinfectant levels over the test period.

(1) Tapwater Test Preparation. Tapwater was dechlorinated by continuous stirring at room temperature overnight. The initial tapwater pH range was 7.2 - 7.4 for all experiments.

(2) Worst Case Water Preparation. Tapwater was dechlorinated by continuous stirring at room temperature overnight. To simulate worst case

water conditions, the following components were added to each liter of test water: 1500 mg of sea salt (Sigma Chemical Co., St. Louis, MO, # S-9883); 10 mg of humic acid (Aldrich Chemical Co., Milwaukee, WI, # dH1,675-2); 150 mg AC fine test dust (Part # 1543094; A.C. Spark Plug Div., GM Corp., Flint, MI) to give a turbidity of 30 NTU. The pH range of these waters before adjustment was 7.4 - 7.6.

(3) Halogen Demand-free Water Preparation. Distilled deionized water was collected in 20 liter glass jugs and chlorinated with Chlorox™ at a concentration of 10 mg/L. The jugs were tightly stoppered and set in the dark for 24 hours followed by exposure to sunlight for an additional 24-48 hours until dechlorination was complete. The pH of the demand-free water was 6.1 ± 0.2.

2. MICROBIOLOGICAL CHALLENGE

Table 2 shows the waterborne microbiological challenges and their removal endpoint requirements. The selection of microbiological challenges differed from that of the USEPA Guide Standard and Protocol[1] in that the challenge for viruses was limited to a single virus, Echovirus 1, to represent the enteroviruses. The use of an Echovirus 1 strain (provided by Dr. Mark Sobsey of the University of North Carolina) in earlier free available chlorine and iodine tablet disinfection kinetics experiments had indicated that this virus was at least as resistant to these halogen disinfectants as Poliovirus 1 and could be considered to be a worst case challenge. Rotavirus was excluded because of its known sensitivity to halogen disinfectants, the nonavailability of the appropriate cell cultures and limitations in the time available. Additionally, the Giardia sp. cyst challenge was replaced in the test protocol by Cryptosporidium parvum cysts as it was felt that the Cryptosporidium represented a worse challenge to chlorine dioxide because of presumed resistance to common water disinfectants. Also, military exposures to Cryptosporidium are thought to be of worldwide significance.

TABLE 2. MICROBIOLOGICAL CHALLENGES AND REMOVAL ENDPOINTS

Test Organisms	Challenge levels/liter	Minimum removal in $\log_{10}$ (%)
Bacteria - Klebsiella terrigena (overnight culture)	10^8 cfu	6 (99.9999)
Enteric virus - Echovirus 1	10^7 pfu	4 (99.99)
Protozoan cysts - Cryptosporidium parvum	10^6 cysts	3 (99.9)

a. Bacteria Test Preparation. Klebsiella terrigena (ATCC #33257) was obtained from the American Type Culture Collection, grown in nutrient broth, and 1 mL portions were frozen at -70°C for seed stock. For testing purposes, a 24-hour culture of Klebsiella at 37°C was prepared from seed stock and

centrifuged at 8000 rpm for 10 minutes using a Sorvall GSA rotor, washed three times in demand-free phosphate buffered saline (PBS), and filtered through a Whatman # 2 filter pad to remove bacterial clumps. The filtered Klebsiella cells were diluted 1:10 in demand-free PBS, and optical density readings were taken using a Klett-Summerson colorimeter (Klett Manufacturing Co., NY). For the challenge water preparation the diluted cell suspension was further adjusted to a colorimeter scale reading of 35 (previously demonstrated to equal approximately 1.00×10^8 cfu/mL) to seed challenge water.

b. Virus Purification and Test Preparation. Echovirus 1, V239 strain (provided by Dr. Mark Sobsey), was replicated in Buffalo Green Monkey Kidney (BGMK) cells (Whittaker Bioproducts, Walkersville, MD), frozen at -70°C and used as seed to prepare the test virus. BGMK cells were grown to a confluent monolayer in Earles' Minimum Essential Medium (EMEM) with 10 percent newborn calf serum in a 150 cm^2 flask, washed two times with 10-ml volumes of PBS, pH 7.2, and infected with 1.0 ml of Echovirus 1 seed at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell. The infected cell culture was rocked every 15 minutes during the 36°C incubation period to insure a thorough mixing of virus for attachment to cells. After 60 minutes a volume of 50 ml of EMEM with 2% fetal calf serum was added to the culture flask. The culture was incubated in 5% CO_2 at 36°C until cytopathic effect (CPE) was around 90% with minimal cell detachment (approximately 12 hours). The medium containing the detached cells was collected in a 150 mL conical centrifuge tube and saved. The infected cell monolayer was washed with 10 mL of pH 7.2 PBS. This wash was combined with the cell culture medium, and the cells were collected by centrifugation at 800 x g for 10 minutes. The supernatant was discarded and the cell pellet was saved. The residual cell monolayer was removed from the flask by addition of 10 mL of PBS followed by scraping with a rubber scraper. These cells were combined with the cell pellet in the centrifuge tube. A second 10-mL volume of PBS was added to the flask to collect any remaining cells, and this volume was removed and combined with the cell suspension in the centrifuge tube. After addition of 1% fetal calf serum, the cell suspension was "freeze-thawed" three times in a dry ice-ethanol bath and then extracted three times in a Waring blender for 1 minute with 1,1,2-trichlorotrifluorethane at a ratio of 4 mL per 6 mL of virus suspension. After each extraction, layers of the mixture were separated by centrifugation at 800 x g for 10 minutes, and aqueous phases were collected and pooled. The aqueous volume containing the virus was concentrated to a final volume of 10 mL in an Amicon Centriprep-30 tube by centrifugation at 1500 x g in an IEC refrigerated centrifuge. The echovirus particles were separated by rate-zonal centrifugation in 10% - 30% sucrose gradients in phosphate-buffered demand-free water (0.05M PO_4 buffer, pH 7.2) at 90,000 x g in a Beckman SW28 rotor for 2 hours and 15 minutes. The gradients were collected in 2 mL fractions and assayed on BGMK cells using the plaque assay titration method. Fractions which contained the highest concentrations of virus, F-9 through F-14, and F-20 were combined to give a proportional number of small, medium and large aggregates.[2] This virus pool contained 2.69×10^9 pfu/mL when assayed in BGMK cells and was used to seed test waters.

c. Protozoan Cyst Preparation. Calf feces (50% in 2.5% potassium dichromate [$K_2Cr_2O_7$]) containing Cryptosporidium parvum oocysts were obtained from the University of Idaho, Department of Veterinary Science, Caldwell, ID,

and partially purified using a modified method of E. Waldman et al.[3] Ten ml volumes of the calf feces suspension were dispensed into 50 mL polypropylene conical centrifuge tubes, and an equal volume of PBS (pH 7.0) with 0.1% Tween 20 [polyoxyethylene (20) sorbitan monolaurate (Aldrich)] was added to each tube. The suspensions in the tubes were mixed well and centrifuged at 750 x g for 15 minutes. The supernatant fluids were discarded, and the pellets were resuspended in 15 mL PBS-Tween 20. Five mL of anhydrous ether were added and mixed with the suspension for one minute. The tubes were then centrifuged at 500 x g for 10 minutes. The top three layers (ether, debris plug and PBS-Tween 20) were removed and discarded. The pellets containing the cysts were resuspended in 10 mL PBS with 0.01% Tween 20 and pooled. Cysts were further purified by filtration through an 8.0 micron porosity polycarbonate filter membrane. The preparation was diluted to contain approximately 1.66×10^6 oocysts/mL for use in the preliminary challenge water test.

3. SAMPLE ANALYSES

a. Chlorine Dioxide Analysis. Chlorine dioxide concentrations were determined using the LaMotte colorimeter (Model MTRL) test procedure. The recommended range with this instrument for chlorine dioxide is 0 to 7.0 mg/L. After addition of chlorine dioxide to the challenge waters, 10-mL samples from each test water were collected in colorimeter tubes (previously rinsed in demand-free water followed by one rinse in water to be tested) at 0.5, 1, 2, 5 and 10 minutes. One 10-ml water sample minus Glycine was used to adjust the colorimeter to 100% transmission for each water condition at the beginning of each test. Five drops of glycine solution (Test Kit # 6811) were added to each test water sample tube and mixed. One N,N-diethyl-p-phenylenediamine (DPD) # 1 tablet (Kit # 6903) was crushed and added to the tube. The tube was shaken until the tablet dissolved and then inserted into the colorimeter chamber. Chlorine dioxide reacts with DPD to form a red color in proportion to the disinfectant concentration. All readings were made within 30 seconds after the DPD tablet was added.

b. Bacterial Analysis. For enumeration, the total coliform membrane filtration procedure of Standard Methods[4] was used, and analyses were conducted according to the USEPA Guide Standard.[1] Prior to chlorine dioxide addition to each challenge test water, 5-mL samples were taken to verify bacterial challenge concentrations. These samples were serially diluted in PBS, and triplicate 1.0-mL volumes of each dilution were filtered through the 0.45 micron porosity bacteriological filters (47 mm diameter). The filters were then placed onto pads saturated with m-Endo broth in 50 mm snap-cap dishes, and incubated at 35°C. After chlorine dioxide was added to each challenge water, 250-mL samples were taken at intervals of 0.5, 1, and 2 minutes after chlorine dioxide addition and immediately added to flasks containing 2.5 mL of 10 percent sodium thiosulfate to quench residual disinfectant. Duplicate 100-mL volumes and triplicate 10-mL and 1-mL volumes were filtered as described above. All colonies were counted after 24 hours incubation at 35°C.

c. Viral Analysis. Five-mL samples of each challenge water were taken prior to chlorine dioxide addition at the beginning of the test and after addition of chlorine dioxide at 0.5, 1, 2, 5, 10, 15, 20 and 30 minutes. The

5-mL samples were immediately added to 5 mL of 2X EMEM containing 2% newborn calf serum and 1% sodium thiosulfate. Subsequent dilutions were made in 1X EMEM containing 2% newborn calf serum. Triplicate 60 mm tissue culture dishes containing 72-hour BGMK cell monolayers were inoculated with 0.25 mL of each virus dilution, incubated 1.5 hours at 36°C, and overlaid with 5 mL of Medium 199 containing antibiotics (5 units/mL Nystatin, 0.05 mg/mL Gentamycin, and 5 units/mL Penicillin-Streptomycin), 2% newborn calf serum, 1.4% Difco purified agar, 0.125% sodium bicarbonate and 0.01 M Hepes buffer. After an incubation period of 48-56 hours in 5% CO^2 at 36°C, 4 mL of Hank's basic salt solution (BSS) containing 5% neutral red stain (GIBCO #630-5330) were added to the the agar surface of each dish, absorbed for 1 hour, and then poured off. Plaques were counted 12 hours after staining.

d. Protozoan Cyst Analysis. To quantify cysts, 100-mL samples of challenge water were removed from each test beaker prior to chlorine dioxide treatment. Thirty minutes after addition of chlorine dioxide, 500-mL volumes were removed from each challenge water beaker and placed in another flask which contained 1 percent sodium thiosulfate. After addition of 0.1 percent Tween 20 to the water samples, the Cryptosporidium oocysts were concentrated by collection on a 1.0 micron polycarbonate filter membrane. Each filter membrane was then cut into four sections and placed into a polypropylene centrifuge tube. Oocysts were collected by washing the filter five times with 10 mL of PBS containing 0.01 percent Tween 20, along with vigorous mixing on a vortex mixer. The oocysts were concentrated from the pooled wash solutions by centrifugation at 1500 x g for 20 minutes. They were then stained for 20 minutes at room temperature with 0.5 percent Malachite Green, decolorized with 0.25 percent sulfuric acid, and counted using the hemacytometer method. Background debris retained the green color, whereas the oocysts were colorless.

4. TEST OPERATIONS

All test challenge waters were contained in 1.5 L halogen demand-free beakers in a water bath connected to a Brinkman RC 20 circulator to maintain test water temperatures. The test water components were kept in suspension by a Bellco multi-stir 9 position magnetic stirrer located beneath the water bath.

a. Chlorine Dioxide Disinfectant Analytical Determination Procedure. Preliminary studies to characterize the chlorine dioxide production from the burst packets covered a range of varying water conditions without microorganisms.

(1) Chlorine Dioxide Analyses Comparison. After consultation with BIO-CIDE International, several methods for the determination of chlorine dioxide residuals were considered, but only two had potential for field use and could determine chlorine dioxide concentrations quickly enough for use in our studies. The spectrophotometric method recommended by BIO-CIDE International reads absorbance at 360 nm and multiplies the reading by the product of the dilution factor X 57.41 to give ClO_2 in mg/L (A360 X dilution factor X 57.41 = mg/L ClO_2). The LaMotte Chemical Co. multiple test colorimeter, Model MTRL, uses a modification of the DPD colorimetric method which reads percent

transmittance at a wave length of 535 nm in the presence of glycine to eliminate interference from free and combined chlorine. The methods were compared using a high concentration of approximately 2000 mg/L and a low concentration of approximately 1 mg/L in demand-free waters with no pH adjustment and no microorganisms. In order to produce chlorine dioxide concentrations of 2000 mg/L for the comparative tests, BIO-CIDE International recommended that 2.0 gm of citric acid be dissolved in 10.0 ml of the sodium chlorite precursor solution in a bulk mixing vessel and allowed to react for 5 minutes. The 1 mg/L concentrations of chlorine dioxide were achieved with a 5-minute reaction time period using the burst pack product.

(2) Characterization of Chlorine Dioxide Concentrations. This experiment was designed to establish a database for chlorine dioxide concentrations in waters of varying temperatures, pH, turbidities, salinities, and TOC levels over a 30-minute contact period. Halogen demand-free and worst case waters were used to represent best and worst case scenarios for disinfectant use. The 1-L volumes of water were adjusted with 1N NaOH or 1N H_2SO_4 to pH 5.0, 7.0, or 9.0. For production of desired concentrations of chlorine dioxide, a 10-minute reaction period was selected and maintained throughout this characterization experiment. The contents of the BIO-CIDE burst packets were mixed, activated for 10 minutes, and then added to 1 L of water. The beakers were covered with parafilm to reduce dissipation of the chlorine dioxide into the air and mixed continuously. Samples were drawn from the beakers at 1, 5, 10, and 30 minutes and analyzed for chlorine dioxide residuals immediately. This process was repeated twice for each water condition.

(3) Reproducibility of Chlorine Dioxide Concentrations. In this experiment, the prototype disinfectant packages were tested by four individual military technicians to determine if the concentration of chlorine dioxide produced was reproducible in the hands of persons not familiar with the test. All subjects were asked to mix the contents in the packet, and then add the resulting disinfectant to 1-L volumes of chlorine demand-free water, which was held at 24°C and covered with parafilm. Their instructions for the production of chlorine dioxide with the burst-pack product were consistent with the method used for the subsequent inactivation/disinfection studies. Instructions for use were as follows: "1) locate end of pack with pleat containing the citric acid solution (this end will be slightly inflated as compared to the opposite end); 2) use thumbs to squeeze the inflated end to force the citric acid activator solution into the other end containing Purogene™ (do not squeeze the solution back and forth from end to end since this causes the contents to foam and become difficult to remove from the packet); 3) after ensuring that all of the activator solution has been displaced, begin timing for 17 minutes and set the burst pack aside; and 4) after the 17-minute reaction period, cut the burst pack in half (just below the membrane separating the upper and lower halves) and squeeze the contents into 1 L of water." Water samples were collected from the 1-L containers only once, at 5 minutes, and analyzed immediately for chlorine dioxide residuals. Each individual performed the test two times.

b. Effect of Chlorine Dioxide on Cryptosporidium Oocysts. A preliminary experiment to microscopically determine the effects of chlorine dioxide on

Cryptosporidium parvum oocysts was performed. Duplicate 1-L beakers of 24°C demand-free water were seeded with approximately 5.0 X 10^6 cysts/liter, and 100 ml water samples were collected from each beaker before the disinfectant was added. Burst packs were activated and added to the test waters to provide 2.0 mg/L of chlorine dioxide disinfectant. The cysts were kept in suspension by continuous mixing on a magnetic stirrer. After a 30-minute contact period, 100 mL samples were again collected, and the chlorine dioxide was inactivated with 1 percent sodium thiosulfate. The cysts were concentrated by centrifugation at 600 x g in an IEC centrifuge for 15 minutes, and the liquid portion was discarded except for 1.0 mL which was used to resuspend the cysts. The concentrated cysts were microscopically examined and counted before and after staining as described in section 3.d above.

c. Chlorine Dioxide Disinfection/Inactivation Studies. These studies examined chlorine dioxide disinfection/inactivation efficacy in halogen demand-free, tap and worst case waters with an unadjusted initial pH of approximately 6.1, 7.3 and 7.4 respectively, and with pH adjusted to maintain 9.0 at 5° and 24°C. The challenge test waters were dispensed into 1.5 L beakers. The adjusted waters were maintained at pH 9.0 by adding 100 mL of borate buffer to each 900 mL of test water. Water temperatures were maintained at 5°C in the refrigerated water bath. Each test condition was run in duplicate along with two controls, one for chlorine dioxide determinations and the other for challenge microorganism concentrations. Microorganisms were added to the challenge waters at the beginning of each test to furnish 2.0-4.0 X 10^7 pfu/L of Echovirus 1 and approximately 1.0 X 10^8 cfu/L of Klebsiella terrigena. Chlorine dioxide was produced using the burst pack with a 15-minute reaction period for the unadjusted pH experiments. A 17-minute reaction period was used for the adjusted pH experiments because the 15-minute reaction period did not produce the required chlorine dioxide concentration of approximately 2.0 mg/L. Components of all challenge waters were kept in suspension by use of a magnetic stirbar.

MATERIALS AND METHODS: COLLABORATIVE STUDY

For this study the USABRDL provided all necessary personnel, supplies, equipment and the chlorine dioxide to perform the actual treatment procedures on waters containing the oocysts as described for inhouse efforts. The study was carried out at the University of Arizona Department of Veterinary Science Laboratories. The Arizona collaborators provided the Cryptosporidium oocysts, neonatal test animals, animal per diem, test animal holding facilities, and reagent waters used in the study. They also determined the appropriate cyst dosages for the infectivity studies, prepared the various disinfectant treated test waters for animal infectivity studies, prepared and infected the animals with control and treated cysts, and performed histological examinations of the gastrointestinal tract of the animals for evidence of Cryptosporidium infection.

1. MICROBIOLOGICAL

a. Cryptosporidium Oocyst Production and Purification. Cryptosporidium oocysts were recovered from the feces of experimentally infected 2-5 day old Holstein bull calves by means of a previously developed method.[5] Briefly, Cryptosporidium parvum infection was produced in calves by feeding 1.0-2.0 x 10^8 infective oocysts suspended in 1 liter of reconstituted commercial milk replacer. The calves were isolated in vealer pens; and the feces excreted during the peak oocyst shedding period were collected, mixed with an equal volume of 5 percent potassium dichromate ($K_2Cr_2O_7$), and stored at 4^0C. The collected feces were sieved sequentially through stainless steel screens of decreasing aperture, ending with 63 micron pore size (230 mesh). Sequential discontinuous sucrose gradient (1.064/1.103 gm/mL) centrifugation followed by isopycnic Percoll™ gradient (1.091 gm/mL) centrifugation completed oocyst purification. The purified oocysts were stored in 2.5 percent potassium dichromate at 4^0C. Oocysts were withdrawn from storage as needed and washed with PBS (0.025M, pH 7.4) by filtration through polycarbonate filters (1-3 micron pore size) or by centrifugation to remove the dichromate storage solution.

b. Cyst Recovery/Concentration From Water Samples. Oocysts were recovered by a combination of filtration and centrifugation procedures. During the experiments, the Cryptosporidium inoculated control and chlorine dioxide treated 1-L samples were filtered through 1 micron pore size Nuclepore polycarbonate membrane filters. Each filter was then carefully removed and placed in a 50 mL tube (tube A) with 10 mL of washing solution (1 L of nanopure water containing 10 microliters of Tween 20) and mixed for 15 seconds. The filter was removed and placed in another 50 mL tube (tube B) with an additional 10 mL of washing solution and mixed. The contents of tube B were added to tube A and the filter in tube B was washed again. The filter in tube B was removed while the second wash from that tube was also combined with tube A. Tube B was washed twice with 7.5 mL of nanopure water and combined with tube A. The entire contents of tube A was centrifuged at 3000 rpm in a Sorvall T-6000B centrifuge with a T1000B rotor for 10 minutes. The liquid portion was aspirated down to 1 mL, and the pellet was thoroughly mixed with the mL of residual wash water. Appropriate dilutions of this concentrated sample were used to measure residual cyst concentrations and diluted for oral gavage of test animals.

Worst case water samples were transferred equally into two 750 mL centrifuge bottles; the original bottles were washed with 10-20 mL of washing solution, and this material was added to the centrifuge bottles. The samples were centrifuged in a T-6000B centrifuge with a T1000B rotor at 3500 rpm for 15 minutes. The liquids were aspirated to a few mL in the bottoms of the bottles, and the pellets were resuspended into the residual fluids. The resuspended materials were transferred into 50 mL tubes and centrifuged again at 3000 rpm for 10 minutes. The fluids were aspirated and the pellets were combined. Finally the residual pellet was centrifuged as before and aspirated to 1 mL. This provided the material used for neonatal mouse dosing.

2. SAMPLE ANALYSES

a. Mice. Late term pregnant female BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Within 24 hours of birth, mouse pups were randomized and placed back with the mothers (6 to 7 pups/litter) to minimize maternal effects on experimental outcomes. Animals were maintained in microisolate cages throughout the experiments. Room temperature was maintained at 18-26°C with a 12-hour light/dark cycle and a relative humidity of 40-70 percent. Mice were fed Teklad™ Mouse/Rat Chow and sterile water ad libitum. Previous experience with this mouse infectivity model indicated that neonatal mice can be routinely infected orally with 10^4 to 10^5 Cryptosporidium oocysts at 5 days of age.[6,7] Gastrointestinal (GI) colonization with oocyst shedding develops 2-5 days later and resolves in about 5-7 days. In a study of susceptibility dynamics infection in relation to the BALB/c mouse, 100 percent of the neonatal mice through 9 days of age were infected when challenged with 10^5 oocysts by oral intubation.[7]

b. Neonatal Mouse Infectivity. Median infectious dose range determinations for the study were conducted by infecting mice by gavage in which a range of Cryptosporidium oocysts were administered to groups of neonatal mice. The ID_{50} dose was determined by extrapolation from the dose range studies in which infection of intestinal villi was determined. The protocol for experimental dosing utilized centrifuged sample preparations from 50 mL centrifuge tubes. The volume was increased to 5 mL (2 mL for centrifuged worst case water) with Nanopure water and mixed to disperse the oocysts. The dilutions needed to obtain the "high dose," "median dose" and "low dose" in 100 microliters of inoculum based on the previously determined ID_{50} and the recovery efficiencies are as follows:

No. of oocyst seeded per liter x (recovery efficiency) divided by (ID_{50} x 10^5) = No. of mL of dilution needed for 10, 000 x ID_{50} in 100 microliters. This was the "high dose".

Dilute the above suspension 1:10 to obtain 1,000 x ID_{50} in 100 microliters. This was the "medium dose".

Dilute the "medium dose" suspension 1:10 to obtain 100 x ID_{50} in 100 microliters. This was the "low dose".

For ID_{50} determinations using untreated oocysts and for experimental studies using oocysts treated with chlorine dioxide, the oocysts (ID_{50} dose and sequential 10-fold higher levels) were administered by oral gavage to 5- to 7-day old neonatal BALB/c mice. This was accomplished with a blunted, slightly bent, 1/2 inch, 25-26 gauge hypodermic needle fitted with a short piece of polyethylene tubing mounted on a 1 cc tuberculin syringe. The animals were sacrificed 7 days post inoculation; and approximately 3 cm of the terminal ileum was removed, fixed in 5 percent formalin, embedded in paraffin, and sectioned. Hematoxylin and eosin stained paraffin sections were examined microscopically for evidence of Cryptosporidium infection in the microvillous region of villous enterocytes. Specimens with parasitic stages present were scored as positive, those without were scored as negative. Positive specimens always showed numerous parasitic stages (at least 50-60 per 100X microscope

field) while no parasites could be found on any sections taken from negative tissue samples. Infection was scored by the relative concentration of Cryptosporidium in the ileum ranging from a 0 to 4+ level of infection. Any level of infection was scored as a positive

3. OOCYST DISINFECTION TEST PROCEDURE

For these collaborative experiments the USABRDL investigative staff prepared the designated test waters (1 L) with the various chemical and physical challenge characteristics and dosed the waters with the oocysts in accordance with the standard test procedure described earlier, with one exception. For these studies, chlorine dioxide burst-type packets were replaced because the contents showed evidence of variability from long term storage. Instead, 0.5 mL of saturated citric acid and 0.5 mL of Purogene™, the BIO-CIDE chlorine dioxide precursor, were measured into separate sterile 1.5 mL polypropylene tubes. The citric acid and precursor tubes were combined and mixed for 30 seconds; and after a 3.5-minute reaction period to give approximately 2.0 mg/L of chlorine dioxide, the mixture was added to the water with a disinfection contact time of 30 minutes. The chlorine dioxide disinfectant levels were monitored in each sample to insure that proper disinfectant levels were attained and maintained. After the appropriate disinfection contact time, the 1-L samples were rapidly neutralized with 1 percent sodium thiosulfate. The samples were then provided to the University of Arizona where the oocysts were concentrated from the treated water, physically enumerated, diluted for infectivity studies and given to the neonatal mice by gavage.

INHOUSE STUDY RESULTS

1. PRELIMINARY CHLORINE DIOXIDE ANALYTICAL CHARACTERIZATION STUDIES

a. Chlorine Dioxide Analyses Comparison. Both analytical methods showed high variability of chlorine dioxide concentrations at higher concentrations, 1951 to 3210 mg/L with the BIO-CIDE method, and 1850 to 3590 mg/L with the MTRL method (Table 3). Although the tests were completed by one operator using the same technique for each repetition, the concentration is relative to reaction time; and manipulation time may have varied slightly from test to test before the reaction forming the ClO_2 could be quenched by dilution in the demand-free water. The variation seen in measured concentrations between ClO_2 analytical methods is probably due to dilution errors in preparing samples for the MTRL colorimeter. Overall, the BIO-CIDE method was comparable with the MTRL method at concentrations between 1500 and 3500 mg/L, but was not able to measure chlorine dioxide concentrations at the low levels of approximately 1.0 mg/L. Further tests at low levels showed the MTRL method was accurate to 0.1 mg/L, which was adequate for our disinfection experiments (0.1-2.0 mg/L).

TABLE 3. COMPARISON OF BIO-CIDE AND MTRL METHODS TO DETERMINE CHLORINE DIOXIDE CONCENTRATIONS

Method	Chlorine Dioxide Concentrations (mg/L) Test 1	Test 2	Test 3	Test 4
High Dose				
BIO-CIDE	3210	2371	1951	2371
MTRL	3590	2170	1850	2780
Low Dose				
BIO-CIDE	BDL[a]	BDL	BDL	BDL
MTRL	0.8	0.9	0.9	0.9

a. BDL = Below detection limit of 1.0 mg/L

b. Characterization of Chlorine Dioxide Concentrations. The mean results of duplicate challenge water tests on the characterization of the burst-pack chlorine dioxide product are summarized in Tables 4 and 5. Water pH represents the pH-adjusted test waters before addition of chlorine dioxide to simulate unbuffered environmental waters which would not resist the drastic pH changes that occur after addition of chlorine dioxide. Chlorine dioxide concentrations were measured at 1, 5, 10 and 30 minutes after addition of the disinfectant. Chlorine dioxide concentrations did not vary within a given challenge water at a specific temperature and pH by more than 0.3 mg/L for most experiments over the 30-minute test period. However, chlorine dioxide concentrations did vary widely between different challenge waters (e.g. demand-free vs. worst case) and within the same challenge water of different temperatures and pH (worst case water at 5°C vs. 24°C and worst case water at pH 7.0 vs. pH 9.0. These concentrations varied from 0.57 to 2.22 mg/L overall. Also, the burst pack-generated chlorine dioxide lowered all the pH levels to 3.3-4.4 regardless of initial pH or water conditions, most likely due to the presence of residual citric acid. Typically, ClO_2 levels in demand-free waters increased over the 30-minute period even though diluted out significantly. On the other hand ClO_2 levels diminished in worst case water with time, thus indicating that the presence of the water constituents either provided a disinfectant demand or hindered the further formation of ClO_2 in the reaction vessel. Maintenance of pH 9.0 was problematic for the determination of ClO_2. Neither test method could detect significant ClO_2 at this high pH, although disinfection of test bacteria and virus was excellent. (The pH alone did not degrade the test organisms.)

TABLE 4. DEMAND-FREE CHALLENGE WATER CHLORINE DIOXIDE LEVELS

Water temperature	Time in minutes	ClO_2 mg/L	Initial water pH	Final water pH
5°C	0	--	5.00	
	1	1.50		
	5	1.57		
	10	1.57		
	30	1.60		3.45
5°C	0	--	7.00	
	1	1.75		
	5	1.95		
	10	1.95		
	30	1.95		3.30
5°C	0	--	9.00	
	1	2.05		
	5	2.22		
	10	2.10		
	30	2.12		3.45
24°C	0	--	5.00	
	1	1.55		
	5	1.61		
	10	1.67		
	30	1.77		3.45
24°C	0	--	7.00	
	1	1.56		
	5	1.65		
	10	1.65		
	30	1.72		3.60
24°	0	--	9.00	
	1	1.35		
	5	1.55		
	10	1.60		
	30	1.61		3.60

TABLE 5. WORST CASE CHALLENGE WATER CHLORINE DIOXIDE LEVELS

Water temperature	Time in minutes	ClO_2 mg/L	Initial water pH	Final water pH
5°C	0	--	5.00	
	1	1.85		
	5	1.90		
	10	1.70		
	30	1.58		3.47
5°C	0	--	7.00	
	1	1.75		
	5	1.70		
	10	1.60		
	30	1.51		3.80
5°C	0	--	9.00	
	1	1.27		
	5	1.30		
	10	1.15		
	30	1.02		4.20
24°C	0	--	5.00	
	1	1.55		
	5	1.47		
	10	1.35		
	30	1.22		3.45
24°C	0	--	7.00	
	1	1.42		
	5	1.27		
	10	1.26		
	30	0.94		3.95
24°	0	--	9.00	
	1	0.75		
	5	0.90		
	10	0.77		
	30	0.57		4.40

c. Chlorine Dioxide Reproducibility Experiment. The results of studies involving use of the ClO_2 burst packs by the four individuals (Table 6) show that three of the four subjects were able to repeat the tests with an individual variation of only 0.1 - 0.25 mg/L in chlorine dioxide concentrations produced from the packets. Overall, average ClO_2 produced from duplicate tests by individuals ranged from 1.05 to 1.80 mg/liter. This indicates variability either in burst-pack constituents or in operator performance.

TABLE 6. VARIATIONS IN CHLORINE DIOXIDE PRODUCTION AMONG INDIVIDUALS

Subject	Chlorine dioxide (mg/L) Test 1	Test 2	Average
1	0.40	1.70	1.05
2	1.50	1.40	1.45
3	1.05	1.30	1.17
4	1.70	1.90	1.80

d. Chlorine Dioxide Effect on Cryptosporidium Oocysts. Microscopic examination of Cryptosporidium parvum cysts after a 30-minute contact time showed no changes in the appearance of the oocyst with 2.0 mg/L chlorine dioxide at a final pH of 3.3 and 23°C. After addition of malachite green stain to the concentrated oocyst suspension for 20 minutes followed by decolorization with 1.0 percent sulfuric acid, oocysts remained colorless, which would indicate that the oocysts were not structurally damaged. Also, no oocyst suture lines or free sporozoites were observed. The concentration of oocysts was 5.44 X 10^6/L initially and 5.38 X 10^6/L after 30 minutes, which also indicates that the cysts remained intact. The collaborative study results of cyst viability after chlorine dioxide contact are discussed below.

2. INACTIVATION/DISINFECTION STUDIES

Results shown in Table 7 indicate that Klebsiella terrigena bacterium is rapidly disinfected (30 seconds) in almost all cases except worst case water at 24°C and pH 9, where 2 minutes were required. Figure 1 compares disinfection levels of Klebsiella in all water conditions. The mean results of duplicate experiments on the inactivation of Echovirus 1 by burst pack produced in 15- to 17-minute reaction periods with all challenge water condition are summarized in Table 8 and Figures 2 through 6. The water pH shown in Table 8 was the final pH taken at 30 minutes. Because the Echovirus detection limit by plaque assay analyses for these experiments was approximately 2.67 X 10^3 pfu/L, some extrapolation was necessary to demonstrate the time and concentration to reach a 4-log removal. This was necessary because the virus challenge levels did not always reach exactly 2.7 X 10^7/L. Experiments using water at 5°C with unadjusted pH (Figure 2) showed the slowest inactivation rates, requiring 10 minutes for each type of challenge water to drop 4 logs in viral titer. Water with unadjusted pH at 24°C (Figure 3) followed, requiring 5 minutes for demand-free water, 2 minutes for tapwater, and 1 minute for worst case water to drop the viral titer below detection limits (BDL). All the tests where pH 9.0 was maintained (Figures 4 and 5) dropped to BDL within 30 seconds with one exception. One of the duplicate experiments with worst case water at 24°C (Figure 5) dropped to the detection limit at 30 seconds but then showed an increase at 1 minute before dropping to BDL at 2 minutes. The bar graph (Figure 6) shows the virus removals with chlorine dioxide in all challenge water conditions. It should be noted that no inactivation of viruses occurred in any of the virus control water beakers with unadjusted pH or with pH maintained at 9.0 for any of the water types and temperatures, which eliminates pH as a factor influencing

bacterial and viral disinfection/inactivation.

TABLE 7. DISINFECTION OF KLEBSIELLA TERRIGENA WITH CHLORINE DIOXIDE

Water Type	Water pH[a]	Water temperature	cfu/L 0 time	cfu/L Midpoint (time, min)	cfu/L Endpoint (time, min)
Demand free	3.3	5^0	$1.43x10^8$	$1.50x10^1$ (1)	$1.65x10^2$ (2)
Tap	4.0	5^0	$2.00x10^8$	$3.25x10^1$ (1)	$1.35x10^2$ (2)
Worst case	4.2	5^0	$1.65x10^8$	$2.00x10^1$ (1)	$5.00x10^2$ (2)
Demand free	3.5	24^0	$1.65x10^8$	---[b]	$<5.00x10^0$ (0.5)
Tap	4.2	24^0	$1.28x10^8$	---	$<5.00x10^0$ (0.5)
Worst case	4.1	24^0	$1.05x10^8$	$2.50x10^0$ (0.5)	$2.50x10^0$ (2)
Demand free	9.0	5^0	$1.63x10^8$	---	$<5.00x10^0$ (0.5)
Tap	9.0	5^0	$1.31x10^8$	---	$<5.00x10^0$ (0.5)
Worst case	9.0	5^0	$1.73x10^8$	$1.00x10^1$ (1)	$5.00x10^0$ (2)
Demand free	9.0	24^0	$1.81x10^8$	---	$<5.00x10^0$ (0.5)
Tap	9.0	24^0	$1.73x10^8$	---	$<5.00x10^0$ (0.5)
Worst case	9.0	24^0	$1.81x10^8$	$2.00x10^2$ (2)	$2.32x10^2$ (5)

a. Water pH was controlled by buffering only for studies showing pH 9.0
b. No midpoint

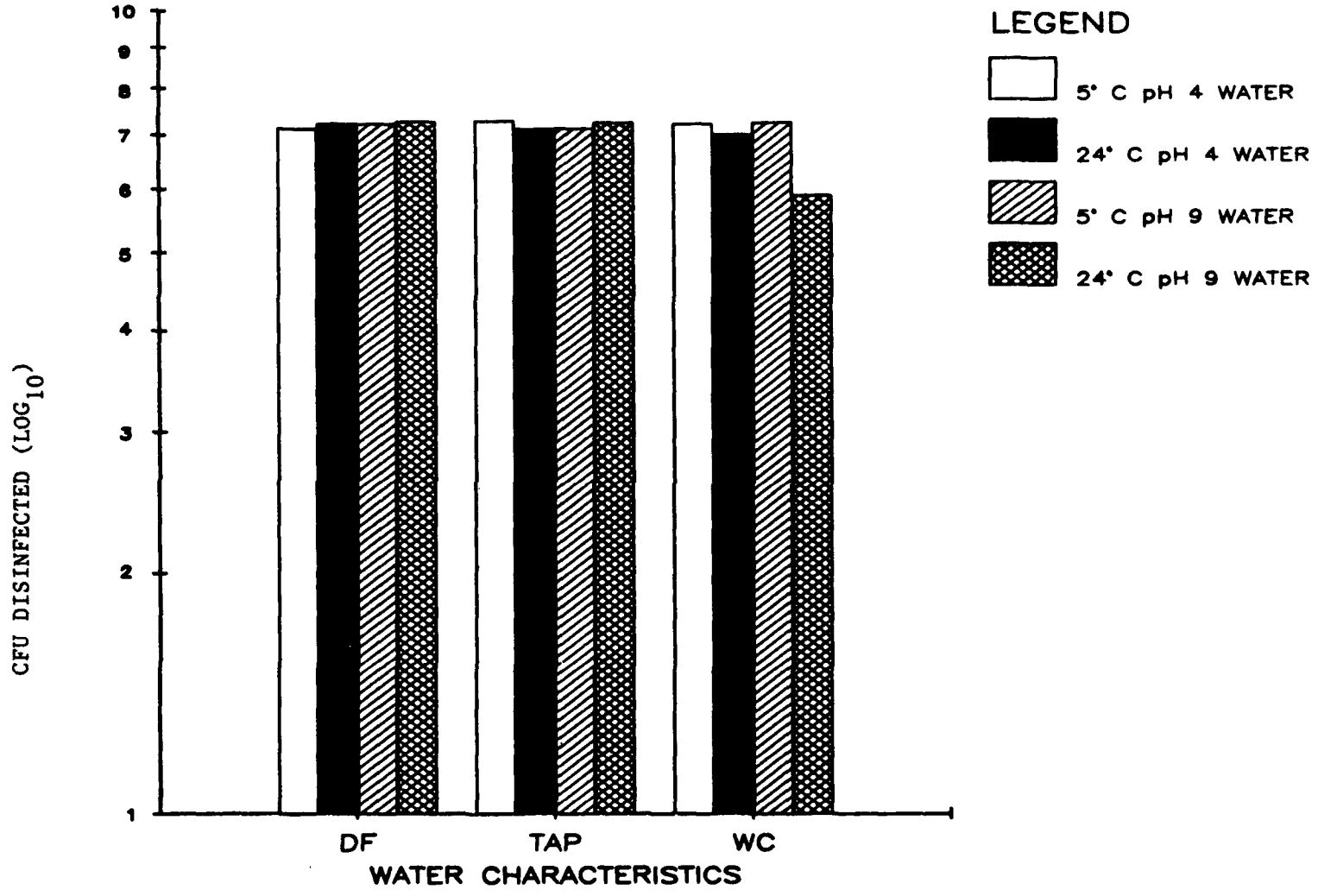

Figure 1. Klebsiella terrigena disinfection with chlorine dioxide (midpoint)

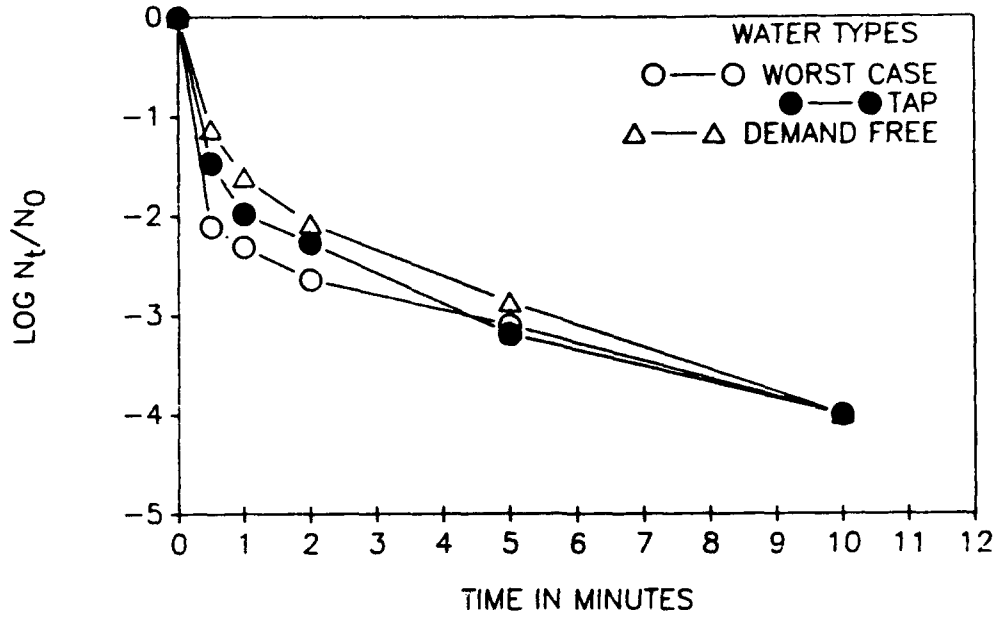

Figure 2. Inactivation of Echovirus 1 by 2 mg/L chlorine dioxide, unadjusted pH, 5°C

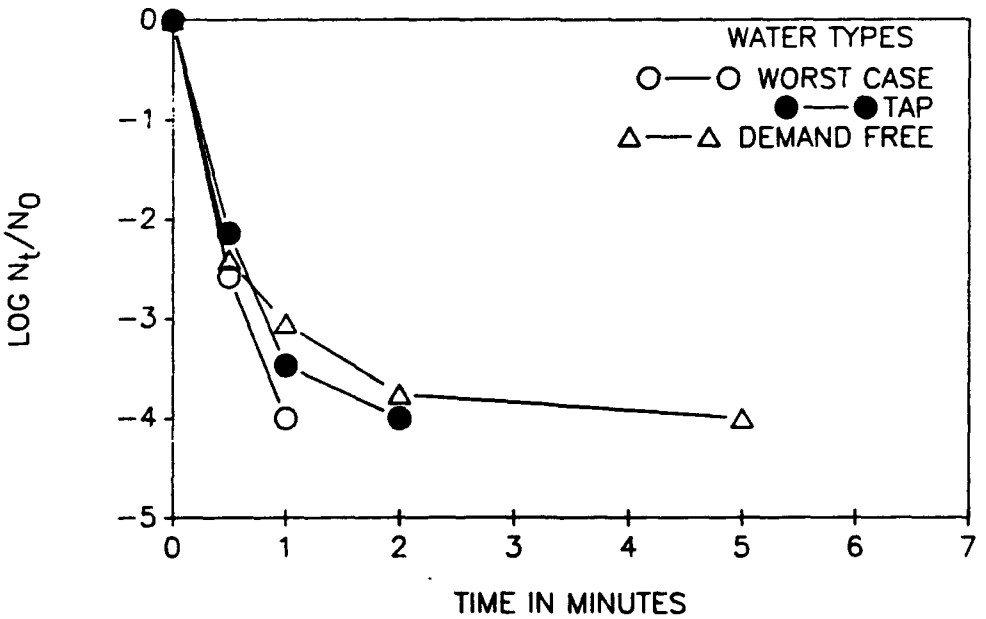

Figure 3. Inactivation of Echovirus 1 by 2 mg/L chlorine dioxide, unadjusted pH, 24°C

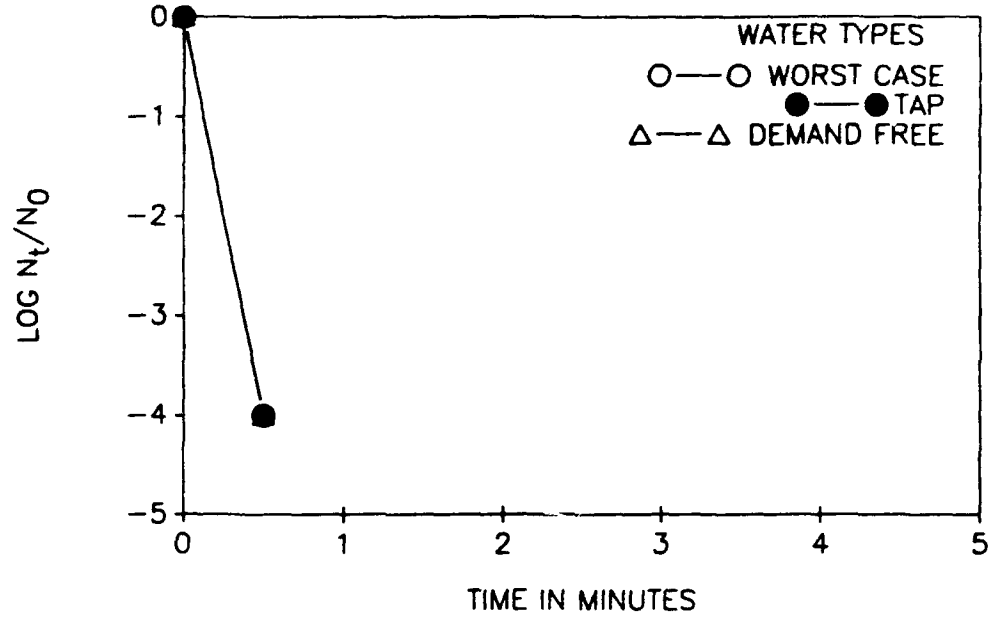

Figure 4. Inactivation of Echovirus 1 by 2 mg/L chlorine dioxide, pH 9, 5°C

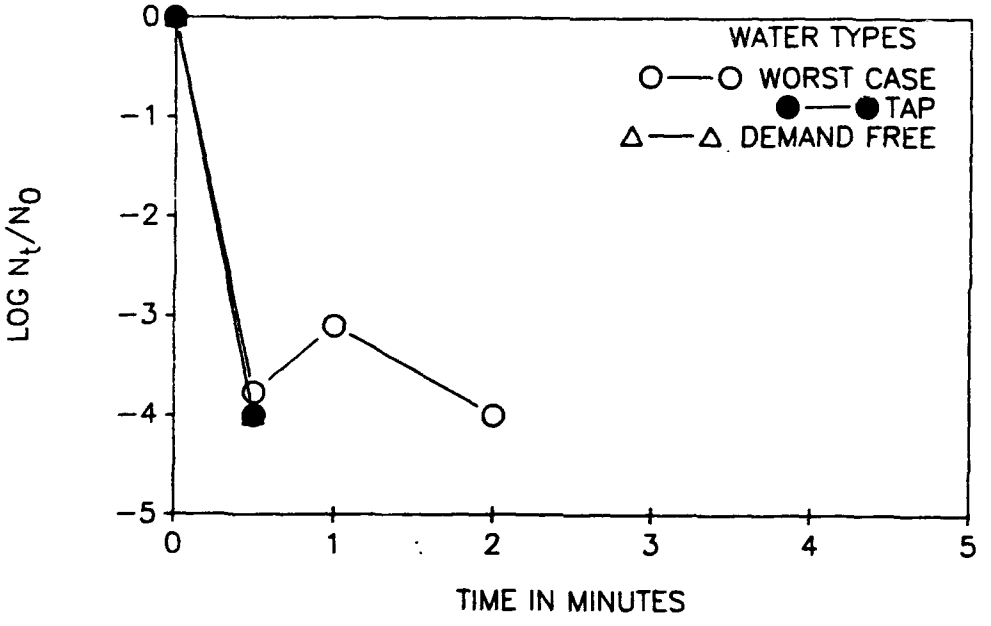

Figure 5. Inactivation of Echovirus 1 by 2 mg/L chlorine dioxide, pH 9, 24°C

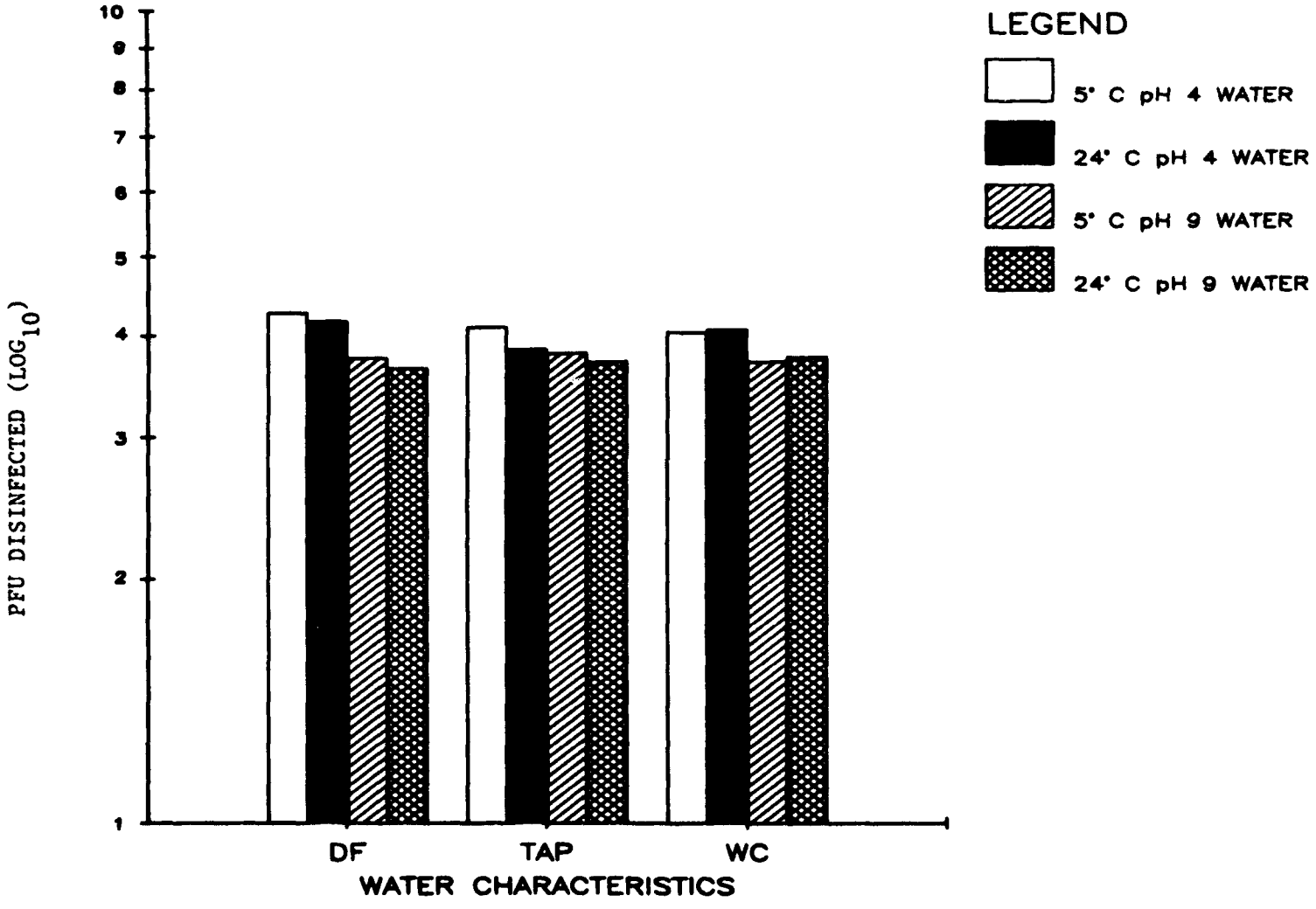

Figure 6. Echovirus disinfection with chlorine dioxide (midpoint)

TABLE 8. INACTIVATION OF ECHOVIRUS 1 WITH CHLORINE DIOXIDE

Water type	Water pH	$[ClO_2]$ 10 min	Water temp.	pfu/L 0 time	pfu/L midpoint (time in min)	pfu/L endpoint (time in min)
[a]Demand free	3.3	1.85	5°	4.90×10^7	6.53×10^4 (5)	$<2.67 \times 10^3$ (10)
[a]Tap	4.0	1.97	5°	3.33×10^7	2.10×10^4 (5)	2.67×10^3 (10)
[a]Worst case	4.2	1.60	5°	4.00×10^7	2.90×10^4 (5)	$<2.67 \times 10^3$ (10)
[a]Demand free	3.5	1.10	24°	3.90×10^7	6.60×10^3 (2)	$<2.67 \times 10^3$ (5)
[a]Tap	4.2	2.10	24°	1.87×10^7	6.65×10^3 (1)	$<2.67 \times 10^3$ (2)
[a]Worst case	4.1	0.93	24°	3.12×10^7	8.10×10^4 (0.5)	$<2.67 \times 10^3$ (1)
[b]Demand free	9.0	0.10	5°	1.45×10^7	---[c]	$<2.67 \times 10^3$ (0.5)
[b]Tap	9.0	0.10	5°	1.70×10^7	---	$<2.67 \times 10^3$ (0.5)
[b]Worst case	9.0	0.15	5°	1.38×10^7	---	$<2.67 \times 10^3$ (0.5)
[b]Demand free	9.0	0.10	24°	1.17×10^7	---	$<2.67 \times 10^3$ (0.5)
[b]Tap	9.0	0.10	24°	1.37×10^7	---	$<2.67 \times 10^3$ (0.5)
[b]Worst case	9.0	0.30	24°	1.54×10^7	---	$<2.67 \times 10^3$ (0.5)

a. Unadjusted pH, 15 min reaction period
b. pH maintained at pH 9.0 with borate buffer, 17 min reaction period
c. No midpoint

COLLABORATIVE STUDY RESULTS

1. MEDIAN INFECTIVE DOSE VALIDATION

Initial studies were performed to validate the median infective dose (ID_{50}) for Cryptosporidium oocysts in the neonatal mouse model. Two groups of 32 neonatal mice 5 to 7 days old were challenged with doses of oocysts which had been exposed to demand-free distilled-deionized water at temperatures of 5° and 10°C for 20 minutes. These data showed that the ID_{50} was 67 oocysts in 5°C water and 80 oocysts for 10°C water. The combined ID_{50} was 72 oocysts. This combined value was used to determine the experimental and positive control challenge doses because the resulting larger group sizes would provide a more reliable measure of the ID_{50} for these oocyst preparations.

2. POSITIVE CONTROLS

Two groups of neonatal mice were challenged with oocysts exposed to each of the test water conditions. One group was given the ID_{50} of 72 oocysts (low dose) while the other received 720 oocysts (high dose). Complete data are shown in Table 9. All mice challenged with the high dose showed a high percentage and level (intensity) of infection. Mice challenged with low doses of oocysts exposed to demand-free distilled-deionized water showed the lowest infectivity while those challenged with oocysts in worst case water showed high infectivity even at the low dose.

TABLE 9. INFECTIVITY OF POSITIVE CONTROLS

Water Temperature and Treatment[a]	Cyst dose	Percent neonatal mice infected
5°C Demand-free	720	100 (6 of 6)
5°C Demand-free	72	43 (3 of 7)
22°C Demand-free	720	100 (7 of 7)
22°C Demand-free	72	67 (4 of 6)
5°C Worst Case	720	100 (6 of 6)
5°C Worst Case	72	86 (6 of 7)
22°C Worst Case	720	100 (8 of 8)
22°C Worst Case	72	100 (5 of 5)
5°C Worst Case pH 9	720	100 (5 of 5)
5°C Worst Case pH 9	72	100 (7 of 7)

a. pH ca. 7 unless otherwise designated

3. EXPERIMENTAL OOCYST DISINFECTION RESULTS

The goal of the test was to determine if there was a significant Cryptosporidium oocyst disinfection capability by the chlorine dioxide when used at recommended concentrations of approximately 2 mg/L. Desired levels of disinfection were 3 orders of magnitude (3 $\log_{10}$). Test waters were seeded with 1.0×10^8 oocysts per liter before chlorine dioxide was added. After the disinfectant contact period was reached the samples were neutralized and concentrated as described earlier (recovery efficiency greater than 99 percent by the method described). Microscopic counts of the final sample concentrate were performed using phase microscopy or monoclonal fluorescent tagged antibody (antibody for the cyst wall) counting procedures to quantify cysts for animal dosing. Two groups of neonatal mice were challenged with oocysts exposed to each of the test water disinfectants. One group was given a "low dose" of 7,200 oocysts (2 logs above the ID_{50} of 72 oocysts) while the other received the "high dose" of 72,000 (3 logs above the ID_{50}). According to this dosing protocol, if any of the mice challenged with the low dose became infected, then a 3-log reduction in cyst infectivity would not have been achieved. This indeed appears to be the case (Table 10). All mice showed unmistakable signs of Cryptosporidium infection except for the low dose of ClO_2 in demand-free distilled-deionized water at 5°C.

TABLE 10. INFECTION OF MICE RECEIVING CHLORINE DIOXIDE TREATED OOCYSTS

Water Temperature and Treatment[a]	Cyst dose	Percent neonatal mice infected
5°C Demand-free	72,000	100 (15 of 15)
5°C Demand-free	7,200	64 (7 of 11)
22°C Demand-free	72,000	100 (11 of 11)
22°C Demand-free	7,200	100 (13 of 13)
5°C Worst Case	72,000	100 (12 of 12)
5°C Worst Case	7,200	100 (12 of 12)
22°C Worst Case	72,000	100 (22 of 22)
22°C Worst Case	7,200	100 (15 of 15)
5°C Worst Case pH 9	72,000	100 (13 of 13)
5°C Worst Case pH 9	7,200	100 (12 of 12)

a. ClO_2 ca. 2 mg/L; pH 3.5 $\pm$ 0.3 unless otherwise indicated

DISCUSSION AND CONCLUSIONS

1. USABRDL STUDIES

The results of the inhouse studies indicate that the use of the burst pack is a viable concept to produce chlorine dioxide in the field on demand. Although chlorine dioxide production for commercial and domestic water treatment typically uses a strong inorganic acid for pH control, citric acid appears to be satisfactory for the burst packs, although it appears that the weaker citric acid requires longer to produce the disinfectant. It was observed over the course of the study that the burst packs lost ClO_2 generation capacity with time. (No disinfectant precursors were observed on the external surfaces of the packs). Improved packs should be considered in order to preclude the loss of reproducibility or even the capability to produce the disinfectant. The ability of a single operator to produce consistent levels of ClO_2 disinfectant appeared to be good when the packets were new, and multiple users also seemed to be able to generate similar amounts of disinfectant with only minor instruction.

Results with the use of the ClO_2 generated from the burst packs appeared to be excellent for either the Klebsiella terrigena bacteria or the Echovirus 1 challenges. Bacteria were typically killed to levels below detection within a minute under all conditions. Likewise, echovirus inactivation generally reached 4 logs when ambient pH conditions were attained during the disinfection process. When the pH was held at pH 9 the inactivation was much more rapid, reaching 4 logs within 30 seconds. Virus maintained at that pH in the absence of disinfectant appeared to survive over 30 minutes without kill. At pH 9 none of the analytical procedures used were able to detect any residual disinfectant during the test. It is not known if the pH bleached out the indicator or whether the buffer somehow interfered with the process. Because of time limitations this was not pursued.

The effectiveness of ClO_2 for disinfecting Cryptosporidium parvum oocysts appeared to be minimal by microscopic examination of the oocyst particles. The cysts were entirely intact after 30 minutes of contact with the disinfectant. There was no overt evidence of any loss of integrity in the oocyst walls, nor was the suture line observable. No sporozoites were observed in any samples.

2. COLLABORATIVE STUDIES

Cryptosporidium infection was noted in the intestinal villi of all test groups of neonatal mice given oral doses of oocysts treated by chlorine dioxide disinfectant. The results demonstrated that there was not a 3-log reduction of oocyst viability through the activity of these chemicals used to treat water as required by the USEPA Guide Standard and Protocol.[1] The high level of infection observed in the groups challenged with the low dose also may rule out a 2-log reduction. There may have been a reduction of less than 2 logs of infective oocysts with chlorine dioxide, but the challenge dose levels used in the tests tend to mask any reduction of lower magnitude. The collaborative test results confirm that chlorine dioxide is not an effective disinfectant for Cryptosporidium oocysts.

The results of these combined studies would thus indicate that chlorine dioxide generated by means of the burst pack would be an excellent disinfectant for destruction of waterborne bacteria and viruses, but would have negligible impact on Cryptosporidium oocysts. The military use of the currently configured chlorine dioxide burst packs would necessitate the additional use of filtration to eliminate particles of oocyst size from the water before consumption.

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APPENDIX A: GLOSSARY OF TERMS

BDL	below detection limits
BGMK	buffalo green monkey kidney
BSS	basic salt solution
cfu	colony forming units
CPE	cytopathic effect
DPD	N,N-diethyl-P-phenylenediamine
EMEM	Earle's Minimum Essential Medium
ID_{50}	infectious dose (50 percent)
MTRL	LaMotte colorimeter
MOI	multiplicity of infection
NTU	nephelometric turbidity units
PBS	phosphate buffered saline
pfu	plaque forming units
USABRDL	U.S. Army Biomedical Research and Development Laboratory
USEPA	U.S. Environmental Protection Agency
WRMAG	Water Resources Management Action Group

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